Virus Particle Counts with the Negative Staining-Spray Droplet Technique.* (28893)

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Various procedures used for electron microscopic counting of virus particles have involved spraying of virus on grids, followed by metal shadowing(1), sedimentation of virus on grids, followed by metal shadowing (2), sedimentation of virus on grids, followed by staining(3), or dialysis of macrodrops on films(4,5), followed by metal shadowing(4). Polystyrene latex has generally been included in the system as an indicator of volume of sample. An additional procedure, which takes advantage of the negative staining-spray droplet technique(6), has been worked out in our laboratory and used routinely for more than a year. Heretofore, the negative staining-spray droplet technique has been applied mainly to studies of virus structure, although recently its use for quantitation was reported (7,8), independent of the present work.

Conditions customarily used for negative staining bring out structural detail, but do not necessarily permit the degree of dispersion of particles or clarity of background which is desirable for purposes of counting. In the present investigation, it was found that varying the concentrations of phosphotungstate and albumin used in the spray mixture had a marked influence on the appearance of the spray droplets. The nature and strength of the buffer or other ionic medium were also of importance. Optimum conditions could be derived from the different findings. Finally, statistical analyses were carried out to establish the degree of reproducibility which could be attained, the observed errors being compared with the theoretical errors predicted by Breese and Trautman(9).

Materials and methods. Test materials used were adenovirus and parainfluenza virus tissue culture fluids and concentrates. The polystyrene latex was Dow Chemical Co. Run No. LS-057-A, having an average particle diameter of 264 mμ. The spray mixture was composed of 0.5 ml of a suitable concentration of virus in 0.02 M pH 7 phosphate buffer, 0.1 ml 3% phosphotungstic acid neutralized to pH 7 with potassium hydroxide, 0.1 ml aqueous 0.8% bovine serum albumin (Armour Fraction V), and 0.1 ml 1/100 dilution of stock latex. In preliminary experiments, described under *Results,* other concentrations and proportions of the various components of the spray mixture were also used. A Vaponefrin vaporizer(10), fitted with a downward-bent right angle elbow of glass tubing, was used for spraying droplets of the mixture onto 200-mesh carbon-coated copper grids. The grids were mounted on a small rubber stopper just below the orifice of the vaporizer. Counts of particles were made directly from the viewing screen of a Philips model 100 electron microscope at a magnification of 6000 $\times$, a reading glass being used to aid in certifying the distinguishing features of the virus particles. Droplets having diameters in the range of 12-24 μ were selected for the counts. For each analysis, 10 grids were sprayed and 20 droplets were examined from each grid. The concentration of virus in terms of number of particles per ml was calculated with the aid of the formula, $n^{\circ}_{v} = n^{\circ}_{1} N_{v} V_{1} F_{1}/N_{1} V_{v} F_{v}$, where n°_{v} represents the number of particles per ml in the original virus sample; n°_{1}, number of particles per ml in the stock latex; N_{v}, number of virus particles counted in the sample of droplets selected; N_{1}, number of latex particles counted in the sample of droplets selected; V_{v}, volume of the aliquot of diluted

* Virus-containing tissue culture fluids used in this study were supplied by Dr. Benjamin H. Sweet and Dr. Louis Potash. Virus concentrates were supplied by Mr. Daniel S. Spicer and Mr. Roy A. Machlowitz. Routine particle count analyses on which statistical calculations were based were carried out by Mr. Jerome Feinberg.

virus sample used; V_l, volume of the aliquot of diluted latex used; F_v, the factor by which the original virus sample was diluted before counting; F_l, the factor by which the original latex stock was diluted before counting. In the present work, V_l was 0.10, F_l was 0.01, and n^o_l, as determined by dry weight measurement and the use of the value 1.052 for the density of polystyrene(11), was found to be 13.73×10^{12}. Under the conditions described, the average count of latex particles per droplet was around 10.

Results. Conditions for staining. The presence of bovine serum albumin in the spray mixture was found to serve the multiple function of delineating the droplets(12), dispersing the phosphotungstate in the droplets, and, as a consequence of dispersing the phosphotungstate, dispersing also the particles of latex and of virus. Decreasing concentrations of albumin tended to cause mottling of the background in the droplets; increasing concentrations lowered the visibility of the particles. Decreasing concentrations of phosphotungstate reduced the mottling but, at the same time, resulted in instability of the stain to the electron beam, producing a bubbling effect. Certain types of mottling of the background improved the staining of virus particles from the standpoint of structural detail; other types of mottling resulted in obscuring of virus particles. The presence of sodium chloride, ammonium acetate, or No. 199 tissue culture medium(13) promoted mottling, while phosphate buffer minimized the effect. Under the final conditions worked out, described above under *Materials and methods,* the degree of mottling was just sufficient to assure adequate staining of virus and latex, and counting of the particles was easy and certain. Difficulty with aggregation of latex particles(7) or of virus was not encountered in the present work. A photograph of a typical droplet is shown in Fig. 1. Virus samples containing media other than 0.02 M pH 7 phosphate were generally dialyzed against the phosphate buffer prior to analysis for particle count. In instances where the concentration of virus in a medium other than the phosphate was high enough that only a very small aliquot was required for analysis, the dialysis step could be eliminated and the virus sample could simply be diluted with the phosphate.

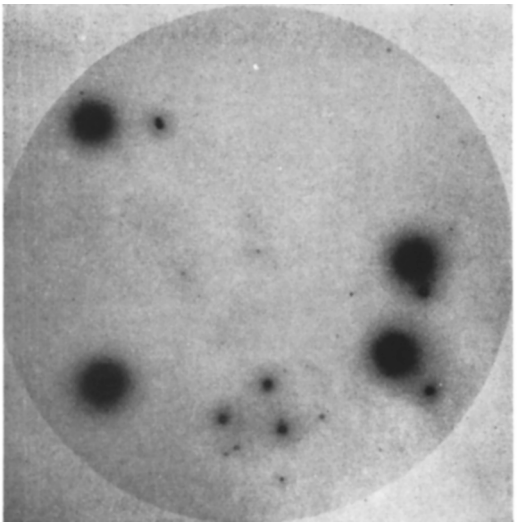
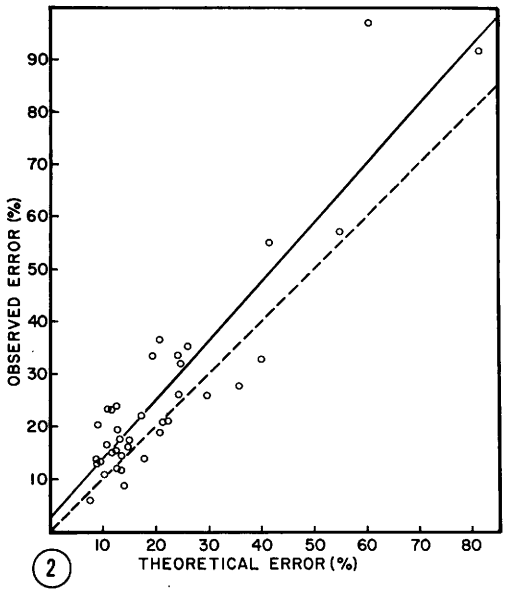


FIG. 1. Photograph of a spray droplet showing latex (4 large, dense particles) and adenovirus (5 small particles with unstained cores).

Reproducibility. Standard errors were estimated from ratios of total counts of virus to latex for replicate grids within analyses. The values obtained, expressed in terms of twice the standard errors, were then compared with theoretical errors, using the expression derived by Breese and Trautman(9), namely, $E = 2(1 + \rho)/\sqrt{\rho N}$, where ρ represents the mean ratio of count of virus to latex; N, total number of particles counted; and E, 2 standard deviations from the mean ratio. The best fitting straight lines relating observed errors to theoretical errors may be seen in Fig. 2 and 3 for analyses of samples of adenovirus and parainfluenza virus, respectively. Exact agreement between observed and theoretical errors would have given the dotted lines which also are drawn in the Figures.

Discussion. The discrepancies between the solid and dotted lines in the Figures are small and the findings provide experimental support for the validity of Breese and Trautman's formula. Factors of technique may be responsible for whatever discrepancies do exist. If the slight differences between the slopes and intercepts of the lines for the adenovirus and the parainfluenza virus are

assumed to be insignificant, averages can be taken and the relation between observed and theoretical error becomes, Obs. = 3.1 + 1.07



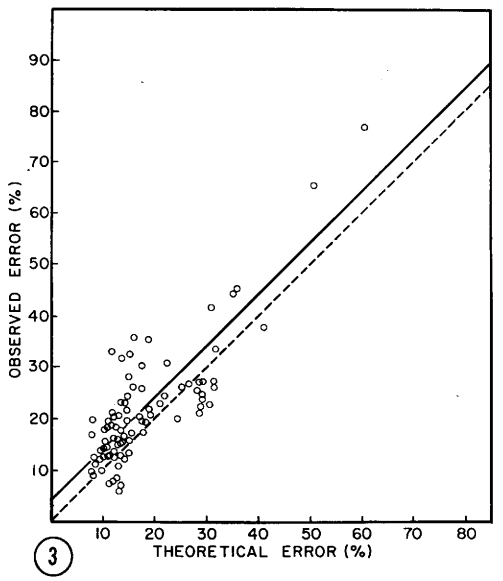


FIG. 2. Relation between observed and theoretical errors of measurement of adenovirus by partical counting method. Solid line is based on points shown; dotted line, on assumed perfect agreement in errors.

FIG. 3. Relation between observed and theoretical errors of measurement of parainfluenza virus by particle counting method. Solid line is based on points shown; dotted line, on assumed perfect agreement in errors.

TABLE I. Relation Between Virus Particle Count, Ratio of Virus Count to Latex Count, and Error of Measurement.

Virus particle count	Virus count / Latex count	% error of measurement	
		Theoretical	Observed
$.28 \times 10^9$	.01	45.2	51.5
.55	.02	32.0	37.3
1.37	.05	20.5	25.0
2.75	.10	14.8	18.9
5.50	.20	11.0	14.9
13.73	.50	7.7	11.3
27.46	1.00	6.3	9.8

Th. Table I shows the relation between virus particle count, ratio of virus count to latex count, and error. The theoretical errors are based on a total of 2,000 latex particles counted and the observed errors are estimated from the equation relating observed and theoretical errors.

The features of the present procedure, which are favorable in comparison with other similar procedures, are (1) direct counting of particles on the viewing screen without necessity of taking photographs, (2) absence of aggregation of latex or of the particular viruses tested, and (3) well-established basis for estimation of errors of measurement. The negative staining-spray droplet technique, as described, is probably the most convenient one for particle counting. It is not, however, as sensitive as those techniques which make use of sedimentation or of macrodrops.

Summary. A procedure for counting virus particles with the electron microscope, using a modification of the negative staining-spray droplet technique, has been described. The experimental errors of measurement exceeded on the average only slightly those predicted by the error formula of Breese and Trautman.

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Increased Responsivity of Adrenals from Reserpine-Treated or Stressed Rats to Steroidogenic Activity of ACTH. (28894)

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In an attempt to determine whether catecholamines serve a permissive role for the steroidogenic action of adrenocorticotrophic hormone (ACTH) in the adrenal cortex, as has been reported for the lipolytic action of ACTH on the fat pad(1), adrenals from reserpine-treated rats were compared to glands from untreated rats with respect to their responsivity *in vitro* to steroidogenic agents. No evidence for a permissive role for catecholamines was obtained in the adrenal system, even when glands from resperine-treated rats were preincubated with acetylcholine and reserpine in an attempt further to deplete residual catecholamines from the adrenals(2).

However, an interesting difference in response to *in vitro* stimulation by ACTH, cyclic 3′,5′-adenosine monophosphate (3′,5′-AMP) or reduced triphosphopyridine nucleotide (NADPH) was shown by adrenals from reserpine-treated rats compared to those from untreated rats. The adrenals of the former group showed a considerably greater production of corticosteroids than those from untreated animals, in response to ACTH or cyclic adenosine 3′,5′-monophosphate (3′,5′-AMP), without change in the steroidogenic response to reduced triphosphopyridine nucleotide (NADPH). This reserpine effect appears to result from non-specific stress activation of adrenals *in vivo*, since injections of formaldehyde or exogenous ACTH reproduced the pattern of *in vitro* response observed following reserpine administration. The results of these investigations are presented here and discussed in connection with current concepts of the mechanism of action of ACTH on the adrenal cortex.

Experimental. Male Sprague-Dawley rats (135-150 g) were used throughout. Groups of animals were treated with one of the following regimens:

Reserpine (Serpasil, Ciba): administered in 2 daily injections for 2 days, 0.1 ml (0.25) mg, intraperitoneally on the first day and 0.05 ml (0.125 mg) intramuscularly on the second day.

When used *in vitro,* reserpine (Mann Research Lab., Inc., New York) was added to the incubation medium as a suspension in KRB to give a final concentration of 1 mg/ml.

ACTH: corticotrophin carboxymethylcellulose (Duracton, Nordic Biochemicals, Inc.); given for 2 days in 3 subcutaneous injections of 0.1 ml (4 U.S.P. units), once on the first day and twice on the second day. For *in vitro* incubations, a pure peptide from sheep pituitaries (α^s-ACTH, obtained from Dr. C. H. Li) was employed.

Formalin stress: a 10% solution of formaldehyde was given subcutaneously for 2 days, 0.1 ml on the first day and 2 injections of 0.2 ml each on the second day.

The response to *in vitro* steroidogenic agents by adrenals from the treated groups was compared with responses obtained in adrenals of untreated rats, which received no injections and were handled as little as possible.